{Exhibit 33}

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Iminobiotin affinity columns and their application to retri val of streptavidin

(avidin/affinity chromatography)

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ABSTRACT A method is described for the retrieval of streptavidin from the culture broth of *Streptomyces avidinii*. The key step in this procedure is the adsorption of streptavidin from culture concentrates to an affinity column in which iminobiotin is attached to AH-Sepharose 4B. This column binds streptavidin at pH 11 and releases the protein at pH 4. The recovery of streptavidin is practically quantitative. The pH dependence of the iminobiotin–avidin affinity, discovered by Green [Green, N. M. (1966) *Biochem. J.* 101, 774–779], has thus found practical application. The streptavidin bound 4.07 ± 0.02 mol of [^{14}C]biotin per mol and was essentially homogeneous as judged by disc and slab gel electrophoresis. Streptavidin was extensively succinoylated without loss of biotin-binding capacity. The observations that ^{125}I-labeled streptavidin and ^{125}I-labeled succinoylstreptavidin are retained by iminobiotin-AH-Sepharose 4B columns at pH 7.5 and are eluted at pH 4.0 provides a convenient purification method for these iodinated proteins. The technique employed for the retrieval of streptavidin is generally applicable to the isolation of iminobiotinylated molecules.

In recent communications (1, 2) we proposed a scheme for the labeling of peptide and protein hormones involving the noncovalent attachment of ^{125}I-labeled avidin of high specific radioactivity to biotinylated hormones. To test this idea a procedure was developed for labeling avidin with ^{125}I to high specific radioactivity ≈2 mCi/nmol; 1 Ci = 3.7 × 10^{10} becquerels. The labeled *p*HPP-avidin‡ bound avidly to rat liver plasma membranes and was not displaceable by unlabeled avidin. Clearly ^{125}I-*p*HPP-avidin was not a suitable label for biotinylinsulin. Because we suspected that the basic nature of avidin (isoelectric point 10.5) could contribute to its affinity for the essentially negatively charged plasma membranes, we subjected *p*HPP-avidin to extensive succionylation with succinic anhydride. This modification did not significantly alter the binding affinity for biotin, but the ^{125}I-labeled Suc-*p*HPP-avidin‡ bound less firmly to the plasma membranes and was displaceable by unlabeled Suc-*p*HPP-avidin. With this material as the label we demonstrated specific binding of $N^{\alpha,B1}$-biotinylinsulin to rat liver plasma membranes. However, the nonspecific binding of the $N^{\alpha,B1}$-biotinylinsulin–^{125}I-Suc-*p*HPP-avidin complex was still considerable. Considering the possibility that the surface carbohydrate on avidin, a glycoprotein, could contribute to its affinity for the plasma membranes, we turned our attention to streptavidin, a biotin-binding protein present in the culture broth of *Streptomyces avidinii*. Streptavidin was isolated in 1963 by Chaiet *et al.* (3), and later Chaiet and Wolf (4) established its chemical nature and determined its amino acid composition. Streptavidin appears to be a 60,000-dalton protein, free of carbohydrate and, lik avidin, consists of four identical subunits of molecular weight of ≈15,000, each containing a single biotin-binding site. Although the biotin binding affinities of avidin and streptavidin are similar, the two proteins differ significantly in their amino acid compositions; however, both are rich in tryptophan. From the point of view of labeling, it is important that streptavidin is reported to contain six tyrosine residues per subunit in contrast to avidin, which contains only a single buried tyrosine per subunit. If, indeed, the carbohydrate on the avidin molecule contributes to the affinity for plasma membranes, then streptavidin may exhibit less nonspecific binding and may have distinct advantages over Suc-*p*HPP-avidin as a label for biotinylated peptide and protein hormones. This reasoning prompted the present investigation.

EXPERIMENTAL

Materials. Sephadex preparations and AH-Sepharose 4B were from Pharmacia. 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate was from Aldrich. *d*-Biotin was a gift from Hoffman–La Roche [^{14}C]Biotin (49 mCi/mmol) was from Amersham. Suc-*p*HPP-avidin was prepared as described (2). Cultures of *S. avidinii* were btained from E. O. Stapley of Merck, Sharp and Dohme and from th American Type Culture Collection (ATCC 27419).

General Methods. The streptavidin content of culture media and concentrates was determined with [^{14}C]biotin according to the method of Wei and Wright (5).

Sodium dodecyl sulfate slab gel electrophoresis was performed in 15% polyacrylamide/0.4% bisacrylamide, using th discontinuous system of Laemmli (6). Disc gel electrophoresis was performed in 7.5% acrylamide gel according to the method of Davis (7).

Iminobiotin Hydrobromide. This compound was prepared from *cis*-3,4-diamino-2-tetrahydrothiophenevaleric acid sulfate derived from biotin (8) by the method of Hofmann and Axelrod (9). The compound was recrystallized from water; mp 222–224°C with decomposition; $[\alpha]_D^{28}$ +57.2° (2.758 g/100 ml in water).

Iminobiotin AH-Sepharose 4B. AH-Sepharose 4B (10 g) was swollen in 0.5 M NaCl and the gel was washed on a Buchner

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Abbreviations: *p*HPP-avidin, 3-(*p*-hydroxyphenyl)propionylavidin; Suc-*p*HPP-avidin, succinoyl-*p*HPP-avidin.

‡ Because avidin contains only one buried tyrosine residue per subunit it cannot be iodinated to high specific activity. In order to render the molecule susceptible to iodination we acylate avidin with the *N*-hydroxysuccinimido ester of 3-(*p*-hydroxyphenyl)propionic acid. The ensuing product, 3-(*p*-hydroxyphenyl)propionylavidin (*p*HPP-avidin), is readily labeled to high specific activity by the chloramine-T method. Succinoylation of *p*HPP-avidin affords succinoyl-3-(*p*-hydroxyphenyl)propionylavidin (Suc-*p*HPP-avidin).

funnel with 0.5 M NaCl (2 l) and water (2 l). The gel was then slurried with water to a volume of 75 ml and iminobiotin hydrobromide (130 mg) was added with gentle stirring. This was followed by 3.2 g of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate, which was added slowly with stirring. The pH was kept at 4.8 by addition of 1% hydrogen bromide. After 2 hr there was little change in the pH and the slurry was stirred slowly for 12 hr at room t mperature. The gel was collected on a Buchner funnel, washed with 2 l of 0.5 M NaCl and 2 l of water, and stored as a slurry (80 ml) in 50 mM ammonium acetate, pH 4.0, containing 0.5 M NaCl (pH 4 buffer). The binding capacity was 1.7 mg of Suc-*p*HPP-avidin per ml of settled gel.

Cultures. *S. avidinii* (ATCC 27419) was grown on plates of medium A (10) plus 2% agar at 30°C. Sporulation appeared after 3–7 days. The cultures were harvested by using liquid medium and a bent glass rod. Harvested spores from one plate were used to inoculate 1 l of medium A. Cultures were incubated at 30°C with mechanical shaking and were harvested after 72 hr of incubation.

Isolati n of Streptavidin from Culture Broth. Four liters of culture broth were centrifuged for 10 min in a Sorvall centrifuge at 10,000 × *g* to remove the organisms. The resulting yellow solution was then concentrated to approximately 400 ml in an Amicon concentrator 2000, using PM 10 ultrafiltration membranes. To the concentrate was added at 0°C solid ammonium sulfate to 70% saturation, and the precipitate was collected by centrifugation for 20 min at 10,000 × *g*. The supernatant was decanted, the precipitate was dissolved in water (approximately 20 ml), and the turbid solution was dialyzed against two 1-liter portions of water for 12 hr. The dialyzed solution was then centrifuged for 20 min at 35,000 × *g*, the supernatant was decanted from the dark sediment, and the pH was adjusted to 11.0 with ammonium hydroxide. An equal volume of 50 mM ammonium carbonate, pH 11.0, containing 0.5 M NaCl was added (pH 11 buffer) and this solution was percolated through a column of iminobiotin-AH-Sepharose 4B (1.5 × 6.0 cm) that had been previously equilibrated with pH 11 buffer. The effluent was collected and assayed for its streptavidin content. Usually none was present. The column was washed with pH 11 buffer until the absorbance of the effluents at 280 nm reached blank values (approximately 150–200 ml). Elution with 50 mM ammonium acetate pH 4.0 (5-ml fractions) released streptavidin, which was detected by absorbance measurements at 280 nm. The contents of tubes containing streptavidin were pooled, dialyzed at 4°C against distilled water (1 liter) for 12 hr, and lyophilized. The streptavidin dissolved in 10% (vol/vol) acetic acid was desalted on a column of Sephadex G-50 (0.8 × 58 cm) and lyophilized. The yield of streptavidin ranged from 10 to 15 mg per 4 l of culture broth. The affinity column was regenerated by washing with 50 ml of pH 11 buffer and was then used for isolation of another batch of streptavidin.

Succinoylstreptavidin. Streptavidin (6 mg, 1 μmol) was dissolved in 0.2 m sodium borate buffer pH 9.0 (8.65 ml), and the solution was cooled in an ice bath. To this solution was added, with stirring, succinic anhydride (4 mg, 40 mmol) in dioxane (0.12 ml), and the mixture was stirred for 1 hr at 0°C. The reaction mixture was dialyzed at 4°C against 10% acetic acid for 12 hr and the dialyzed solution was lyophilized. The residue, dissolved in 10% acetic acid, was passed over a column (0.8 × 55 cm) of Sephadex G-50 and lyophilized; yield was ≈6 mg.

Iodinati n f Streptavidin and Succinoylstreptavidin. These proteins w re iodinated (specific activity ranged from 0.9 t 1.4 mCi/nmol) as described for the iodination of Suc-*p*HPP-avidin (2). The reaction mixture was passed through a Sephadex G-50 column (0.9 × 54 cm) in 50 mM Hepes, pH 7.5, and fractions (1.5 ml) wer collected and their radioactivities were measured. Fractions corresponding t the pr tein peak were pooled and frozen at −70°C. The iodinated proteins were retained by iminobiotin-AH-Sepharose 4B columns (0.5 ml of settled gel at pH 7.5). The columns were washed ext nsively with 50 mM Hepes, pH 7.5, containing 0.5 M NaCl. Th labeled proteins were released from the column with 50 mM pH 4.0 sodium acetate containing 0.5 M NaCl.

RESULTS AND DISCUSSION

In 1968 Cuatrecasas and Wilchek (11) described a single-step purification of avidin from egg white by chromatography on a biocytin-Sepharose column. The avidin was eluted from the affinity gel with 6 M guanidinium chloride at pH 1.5. We attempted to purify a sample of streptavidin, received from Merck, Sharp and Dohme, by this rather drastic procedure with discouraging results. A superior method for the isolation of streptavidin from culture broth of *S. avidinii* was required, and such a method is described in this communication. In 1950 Hofmann and Axelrod (9) synthesized iminobiotin (Fig. 1) and found that this guanido analog of biotin failed to support growth of a number of biotin-requiring organisms. Green (12) investigated the thermodynamics of the binding of iminobiotin to avidin and determined a pK of 11.9 for the guanido group of the biotin analog. Importantly, he indicated that the free base of iminobiotin was the species that binds to avidin and that the iminobiotin–avidin dissociation constant was pH dependent, increasing with decreasing pH. These fundamental observations suggested to us that iminobiotin affinity columns could be expected to bind streptavidin at high pH values and would release the protein on elution with buffers of low pH. Accordingly, we prepared an affinity column in which iminobiotin was attached covalently to AH-Sepharose 4B. This column bound Suc-*p*HPP-avidin regardless of whether it was applied at pH 7 or pH 11. Elution with a pH 4 buffer of the extensively washed column resulted in a practically quantitative release of the retained Suc-*p*HPP-avidin. Encouraged by this result, we applied a streptavidin concentrate to the iminobiotin column in pH 11 buffer and were able to isolate highly purified streptavidin by elution with an acetate buffer at pH 4. The retrieval of the streptavidin was essentially quantitative. The streptavidin bound 4.07 ± 0.02 mol (mean ± SD) of [^{14}C]biotin per mol and exhibited a single, diffuse, band on disc gel electrophoresis. On slab gel electrophoresis, under denaturing conditions, it formed a sharp band corresponding to a subunit molecular weight of ≈15,000; some minor impurities were present. In view of the marked effect of succinoylation on the pHPP-avidin–plasma membrane interaction (2), we subjected streptavidin to extensive succinoylation. The modified protein bound 3.08 ± 0.12 mol of [^{14}C]biotin per mol of streptavidin. This lowered binding capacity could be attributed to inhomogeneity of the succinoylated product, which may contain components of various degrees of succinoylation and affinity for biotin However, further study will be necessary to clarify this point.

Both streptavidin and succinoylstreptavidin are readily io-

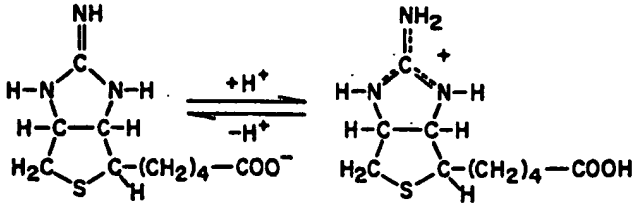

FIG. 1. Structure of iminobiotin.

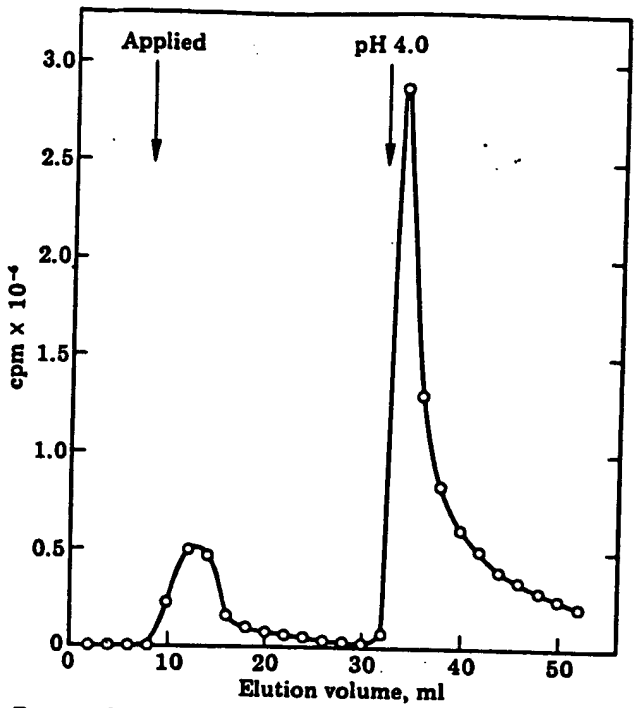

FIG. 2. Chromatography of ^{125}I-succinoylstreptavidin on iminobiotin-AH-Sepharose 4B.

dinated by the chloramine-T method to high specific activity. The observation that the iodinated proteins bind to iminobiotin-AH-Sepharose 4B at pH 7.5 and are released at pH 4.0 provides a convenient purification procedure and demonstrates that iodination does not destroy the affinity of streptavidin and its succinoyl derivative for iminobiotin (Fig. 2). In this connection it is of interest to note that these molecules exhibit significant affinity for immobilized iminobiotin at pH 7.5, at which the iminobiotin is already largely protonated. On the basis of a pK of 11.9 for the guanido function of iminobiotin, the ratio of the protonated to the unprotonated form at pH 7.5 can be calculated to be 25,000:1.

The pH dependence of the iminobiotin–avidin dissociation constant (12), as discussed previously, adds dimensions to the avidin–biotin technique as evidenced by the results communicated in this paper. Variants of the technique employed to retrieve streptavidin are obviously generally applicable to the retrieval of iminobiotinylated molecules. We are particularly concerned with the use of this system for the isolation of polypeptide and protein hormone receptors.

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